AWARD NUMBER:
W81XWH-15-1-0385

TITLE:
Reprogramming the Metastatic Microenvironment to Combat Disease Recurrence

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REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2017	Annual	30 Sep 2016 - 29 Sep 2017

4. TITLE AND SUBTITLE
Reprograming the Metastatic Microenvironment to Combat Disease Recurrence

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0385

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The prognosis for patients with widespread metastatic breast cancer is dismal and for many of these patients current tumor targeted therapies are not curative. Therefore, the development of new clinical approaches that are effective at preventing and/or treating metastatic BC is of paramount importance. One such promising approach is by "reprograming" the tissue microenvironments that provide "safe harbor" for disseminated tumor cells during adjuvant therapy. Our approach to destroying these "safe harbors" is to modulate the patient's immune system. If we could reawaken the immune programs that destroy tumors, especially during adjuvant therapy when tumor cells are most vulnerable, we could truly eliminate "residual disease" and prevent metastatic recurrence. We believe we have found a way to accomplish this by inhibiting colony-stimulating factor-1 receptor (CSF1R) with clinically available therapeutics.

15. SUBJECT TERMS
metastatic breast cancer, immune surveillance, bone, dendritic cells

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	12	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

1. Introduction.

This research aims to "eliminate the mortality associated with metastatic breast cancer". We will do so by: **1)** testing a novel combination of clinically available therapeutic agents that modulate the metastatic tumor microenvironment for the prevention of metastatic disease and **2)** using existing breast cancer patient samples to create a biomarker panel that combines disseminated tumor cell (DTC) classification and "immune-subtyping" to identify those patients most likely to benefit from this new treatment approach.

In spite of recent advances in patient stratification and the use of targeted adjuvant therapies, 10-20% of patients with invasive tumors will eventually develop metastatic disease[1,2]. These data indicate that for a significant percentage of patients, adjuvant therapies are ineffective at eliminating DTCs, which give rise to life threatening metastatic lesions. Therefore, the development of new clinical approaches that are effective at preventing metastatic breast cancer (BC) is of paramount importance. One promising approach is to "reprogram" the tissue microenvironments that provide "safe harbor" for DTCs during adjuvant therapy. Bone is one such metastatic "safe harbor." Sixty-seven percent of metastatic patients will develop disease in the bone[3], which has been proposed to be a reservoir of malignant cells destined for subsequent metastatic relapse in the bone and visceral organs[4]. Interactions between DTCs and the bone microenvironment lead to pathological bone loss, which can stimulate tumor cell outgrowth. In addition to contributing to morbidity, this 'vicious cycle' also protects tumor cells from the cytotoxic effects of chemotherapy[4]. This protective microenvironment may also create an immune privileged site that prevents immune surveillance[5] and effective immunotherapy. To better target bone metastasis with immunotherapy, we have been examining how T cell immune surveillance and antigen presentation affect metastatic relapse in the bone. In so doing, we discovered that inhibiting colony-stimulating factor-3 (CSF3), which blocks pathologic activities of osteoclasts (OCs), serendipitously also increases antigen presentation by CD103^{+} dendritic cells (DCs). We hypothesize that thru these activities CSF1R blockade can destroy the "safe harbor" within the BM, leaving DTCs sensitive to attack by immunotherapy. We will test this using animal models and human clinical samples.

A. The tumor immune microenvironment regulates outcomes. The exact composition of immune cells in primary breast tumors is a significant predictor of chemotherapeutic responsiveness and recurrence-free survival[6-9]. In particular, the presence and activation status of tumor specific T lymphocytes is strongly associated with better clinical outcomes[6-9]. Unfortunately for many patients, this is not the dominant immunologic response[7,10]. Instead many patients have significant numbers of tumor-infiltrating innate immune cells including macrophages and immature dendritic cells[11-14]. In addition to instigating immune suppression, these tumor-infiltrating myeloid cells can actively promote resistance to cytotoxic therapies and the survival of DTC at the sites of future metastases [5,7,15-19].

One critical regulator of the pro-tumor activity of myeloid cells is signaling through the receptor tyrosine kinase CSF1R[7,20,21]. In primary mammary tumors, CSF1R signaling is critical for the recruitment, survival, and pro-tumor activities of monocytes and macrophages[7,22-24]. Previously, we demonstrated that inhibition of CSF1R improves the efficacy of neoadjuvant chemotherapy murine mammary tumor models[7,19,25]. While CSF1R inhibition decreased the number of tumor-associated macrophages (TAMs), surprisingly it also restored anti-tumor T cell activity[7,21,26]. This increase in CD8^{+} cytotoxic T lymphocytes (CTLs) was necessary for CSF1R blockade to enhance chemotherapeutic efficacy and restrain metastasis[7,21]. These provocative findings led our pharmaceutical collaborators to launch a phase I clinical trial of CSF1R inhibition in combination with chemotherapy in locally recurrent breast cancer (NCT01525602).

B. Targeting CSF1R signaling improves immunotherapeutic response and augments CD103^{+} DCs. Recently, in pancreatic cancer models, we showed that CSF1R blockade improves immunotherapeutic efficacy in part by increasing antigen presentation by CD11b^{low}CD103^{+} DCs ([20]

and not shown). The enhancement of CD103^{+} DCs by CSF1R blockade is a novel finding and of critical importance to tumor immunity. This DC subset is critical for sustaining T cell-mediated immune surveillance by antigen cross-presentation to CD8^{+} T cells in breast tumors[19,27].

2. Keywords:
metastatic breast cancer, immune surveillance, bone, dendritic cells

3. Accomplishments:
3A. Accomplishment on the major goals
(Please note all data figures are attached to the end of this document.)
Aim 1. Major Task 1. Determine the efficacy of CSF1R inhibition in combination with chemotherapy and immunotherapy in mouse models of established metastatic disease: Our assessments of bone metastatic models suggest that neutralization of CSF1R can modestly improve responses to chemo or checkpoint immunotherapy (**Figure 1A-C**). These data while suggesting that neutralizing CSF1R could be an effective approach, but also suggest other factors may compensate for CSF1R neutralization to enforce immune suppression. To further these studies we will investigate these factors as well (see below)

Aim 1. Major Task 2 Determine if CSF1R blockade improves T cell responses in bone metastases by impacting DC subsets. Our investigation in this area found that 1) mature CD24^{+} and SIRPα^{+} conventional dendritic cells (cDCs) number are dramatically down-regulated in the bone marrow of tumor bearing and bone metastatic mammary tumor models (**Figure 2A-G**). We have also discovered that this down-regulation of cDCs is likely due to a change in the differentiation of these cells in the bone marrow. These data were demonstrated using adoptive transfer of DC-progenitor cells in tumor bearing and tumor naïve mice (not shown). The result of this lack of cDC differentiation is impaired baseline and induced tumor immunity (**Figure 2I**).

As explained in last years report we have also expanded our analysis to include CSF3/CSF3R signaling. Using this approach we have found that neutralization of CSF3/CSF3R signaling is even more potent than CSF1R inhibition at increasing CD24^{+} cDC (**Figure 3A**). We found that CSF3 inhibition results in restoration of both developing and fully differentiated CD24^{+} cDC1 numbers (**Figure 3B**). CSF3 can also down-regulates cDC1 differentiation directly in in-vitro assays (**Figure 3C**). Additionally both in-vivo and in-vitro the mechanism of action by which CSF3 down regulated cDC1 differentiation is through decreasing the IRF8 transcription factor (**Figure 3C**). We also found that CSF3 is produced by tumor cells in both human and mouse breast tumors and knockout of CSF3 specifically in the tumor cells restored developing and fully differentiated CD24^{+} cDC1 numbers in the BM and periphery (**Figure 4A-C**). Together these data suggest CSF3 is a major regulator of cDC1 differentiation and tumor immunity.

Aim 2. Major Task 1
We have collected and analyzed a total of 45 bone-marrow samples and matched blood samples from human breast cancer patients. We have also immune profiled the majority of these samples by high density FACS and compared them to samples from "healthy donors". Our findings are as follows: 1) Several populations of dendritic cells and their pre-cursors are down regulated in cancer bearing patients compared to normal controls (**Figure 5A-C**). 2) The relative number of bone marrow CD141 DCs in the bone marrow of patients correlates with increased response to neo-adjuvant chemotherapy and decreased metastatic recurrence (**Figure 5D**). 3) Circulating numbers of DC progenitors are significantly down-regulated in breast cancer patients blood (**Figure 5C**). We also found that IRF8 is down regulated in cDC1 progenitors in cancer bearing individuals and the extent of down regulation predicts recurrence free and overall survival (**Figure 5F-H**).

Aim 2. Major Task 2

In part based on our CSF1/CSF1R research a new study of CSF1 neutralization in combination with chemotherapy in breast cancer patients (NCT03285607) has opened at WUSM and we are a sub-Investigator.

3B. Dissemination of these findings.

These findings were presented as a poster at the Keystone Symposium on "Cancer Pathophysiology: Integrating the Host and Tumor Environments (C3)" in Breckenridge, Colorado March 28th as well as a podium talk at the 2017 San Antonio Breast Cancer Symposium. A publication has been submitted for publication in *Nature Communications* and we are waiting final review decision.

4. Impact

"Nothing to report."

5. Changes/Problems

5A. Changes in approach, additional approaches proposed based on new data:

We are and have expanding Aim1A and Aim1B to include the neutralization of signaling through CSF3/CSF3R. This was originally proposed as an alternative but likely plays a significant role in DC development and immunotherapy response. We will evaluate this in addition to current CSF1R work.

No changes to human, vertebrate animal, or biohazard compliances.

6. Products

"Nothing to report." This is year 1.

7. Participant and other collaborating organization

Name:	David DeNardo
Researcher Id (ERA commons)	ddenardo
Nearest person months	1 month
Contribution to Project	Project PI
Funding source	DOD, NCI, Departmental funds

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Funding source	Cancer Biology Pathway Fellowship, DOD

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Researcher Id ()	N/A
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Contribution to Project	Research Technician
Funding source	NCI, DOD

Name:	Rabecca Aft
Researcher Id (ERA commons)	rabaft

Nearest person months	0.5 month
Contribution to Project	Co-Investigator
Funding source	NCI, DOD

## 8. Special Interest
"Nothing to report."

DOD-Report 1 Figure 1

A Leg Bone Tumor Burden by BLI

**B** Bone Tumor Burden

C CD8+ CTLs
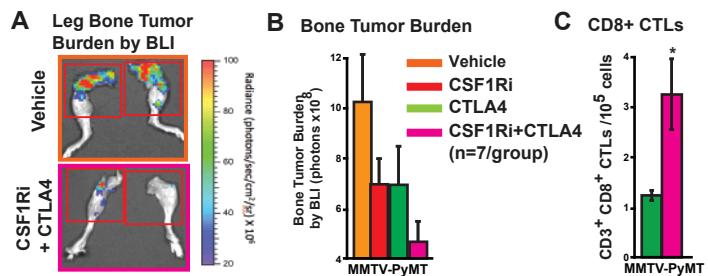
# Figure 2. Primary mammary and pancreatic tumors systemically decrease cDC1s


a. Bone Marrow
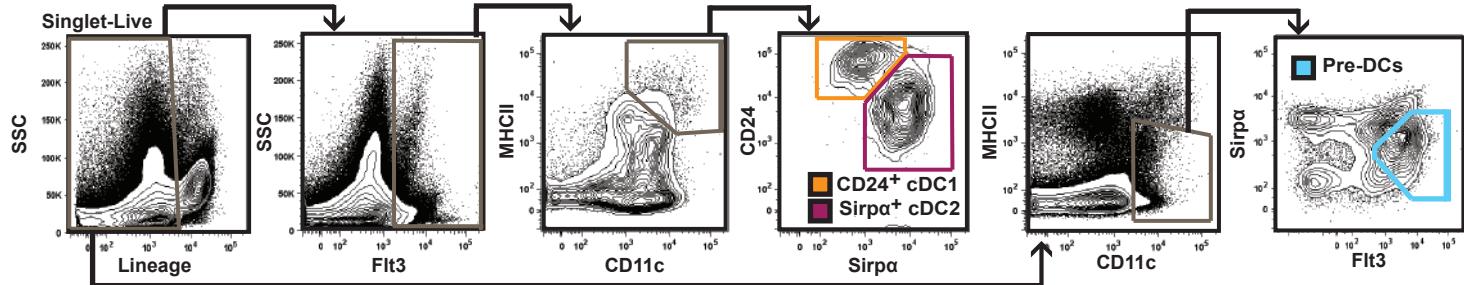
b. PyMT-B6 bone marrow
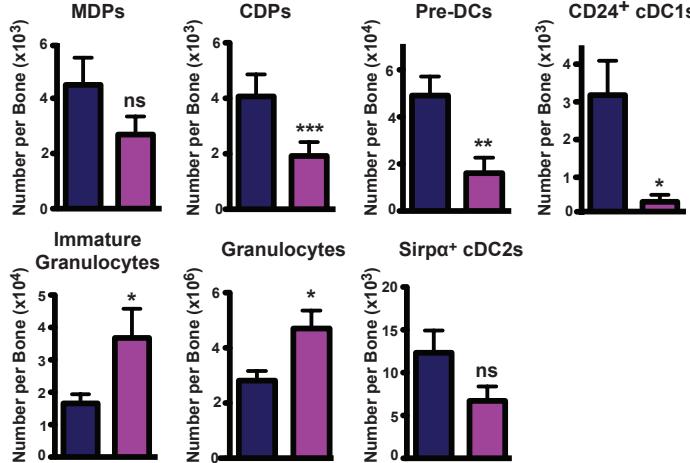
c. Genetic mouse models bone marrow
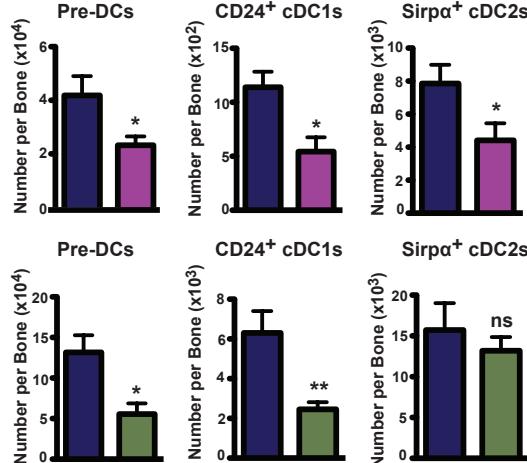
d. PyMT-B6 bone marrow
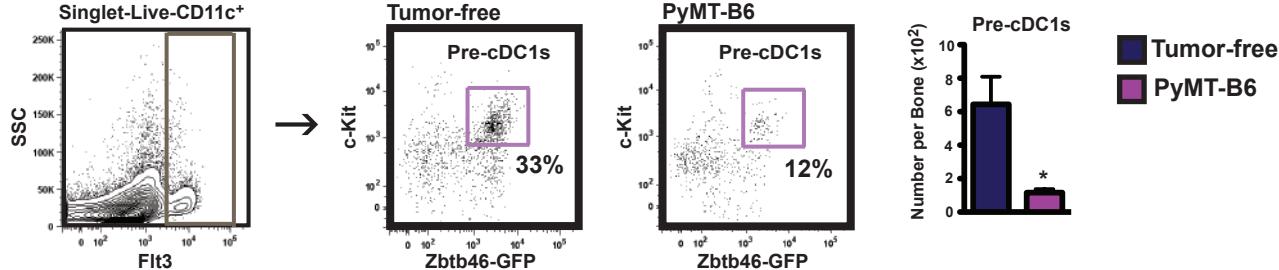
e. Blood
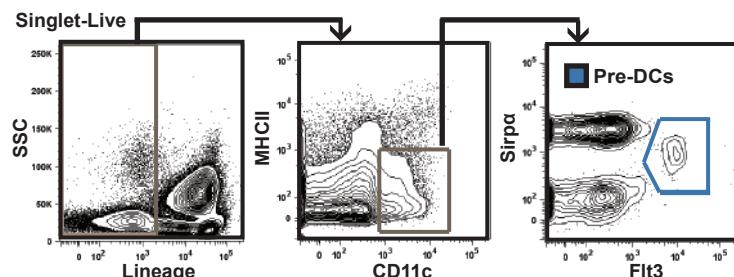
f. PyMT-B6 blood
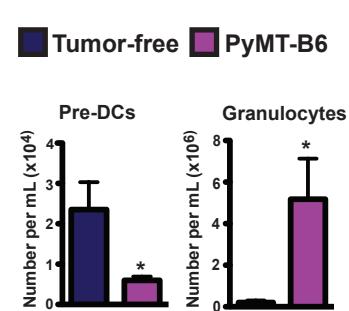
g. Genetic mouse models blood
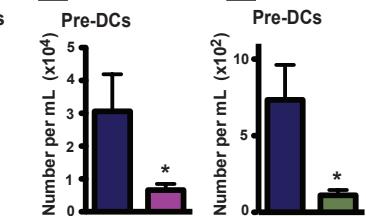
h. PyMT-B6 lymph node
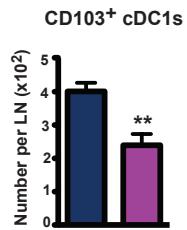
i. *De novo* cDC1 recruitment
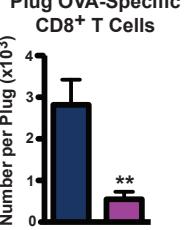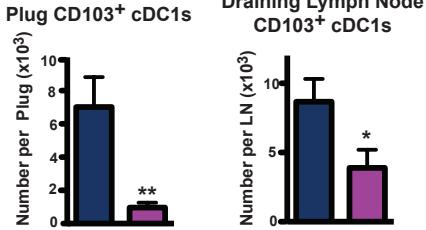
DOD-Report 2 Figure 3

A. CSF1R blockade

BATF3-Dependent Dendritic Cells
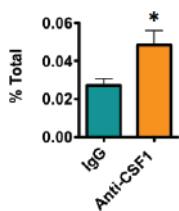
b. GCSF neutralization *in vivo*

14 days — Analysis

50ug Anti-GCSF

■ Tumor-free ■ PyMT-B6 ■ PyMT-B6 + Anti-GCSF

Bone Marrow

Pre-DCs — CD24+ cDC1s — Immature Granulocytes

Blood

Pre-DCs — Granulocytes

Tumor

CD8+ T Cells
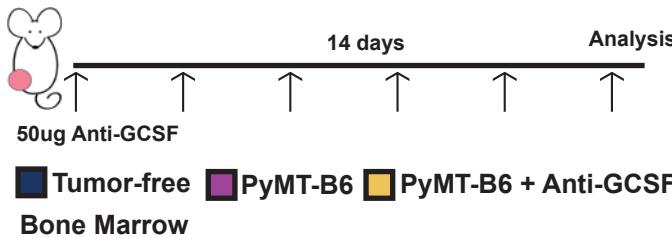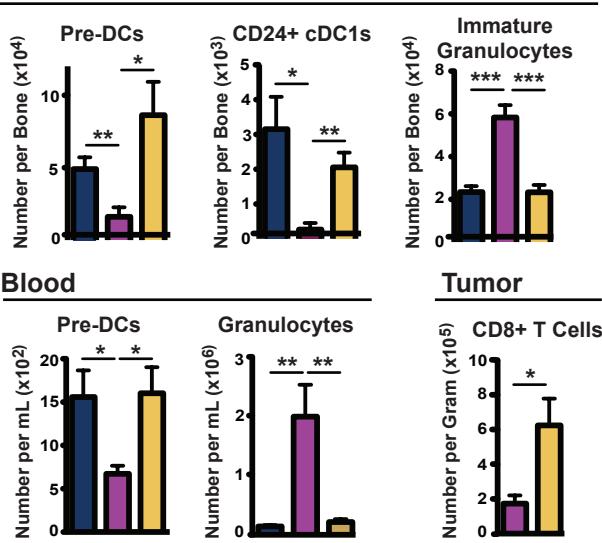
c. *In vitro* cDC1 differentiation

CD45.2+ MP MDP or CDP → CD45.1+ Bone Marrow Feeder Layer + Cytokines

■ Flt3L ■ GCSF ■ Flt3L+GCSF

MPs → cDC1s   MPs → Granulocytes

MDPs → cDC1s   CDPs → cDC1s
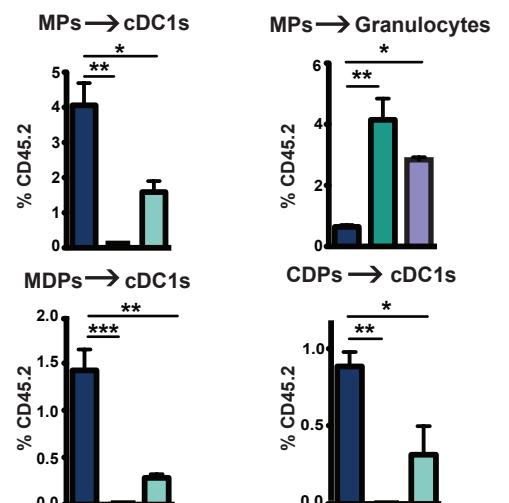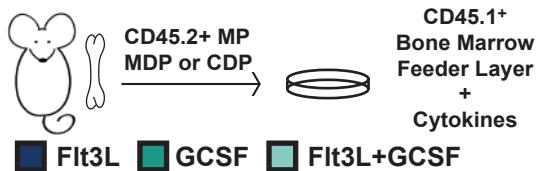
d. IRF8 expression PyMT-B6 bone marrow

■ Unstained ■ Tumor-free ■ PyMT-B6 ■ PyMT-B6 + Anti-GCSF

MDPs   CDPs   Pre-DCs

IRF8   IRF8   IRF8
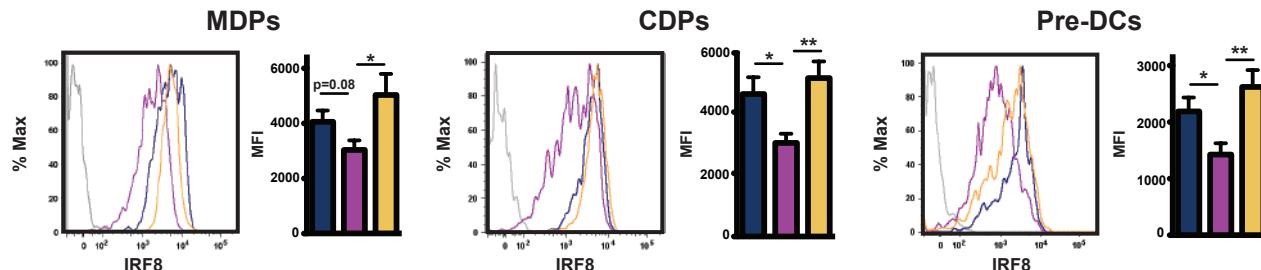
DOD Figure 4

a. Mouse
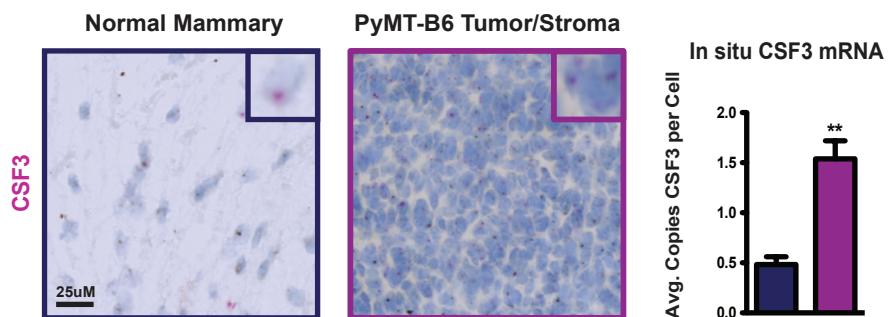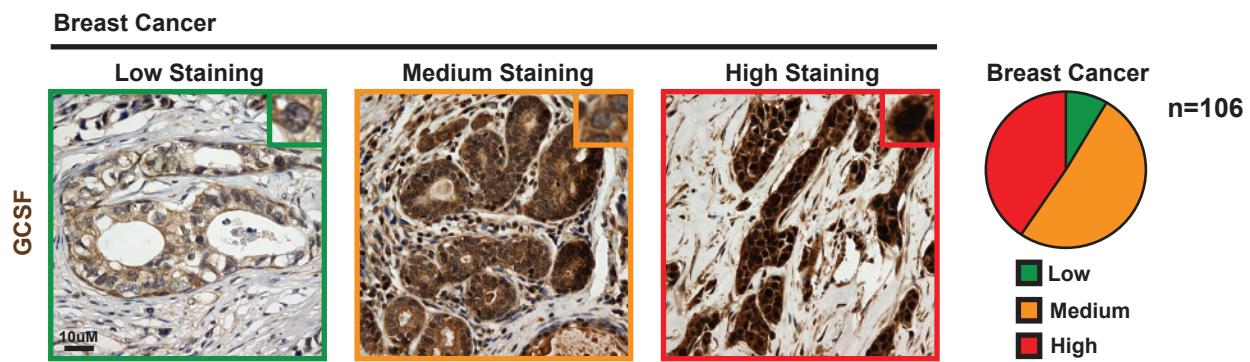
b. Human Patient

## c. GCSFKO PyMT-B6
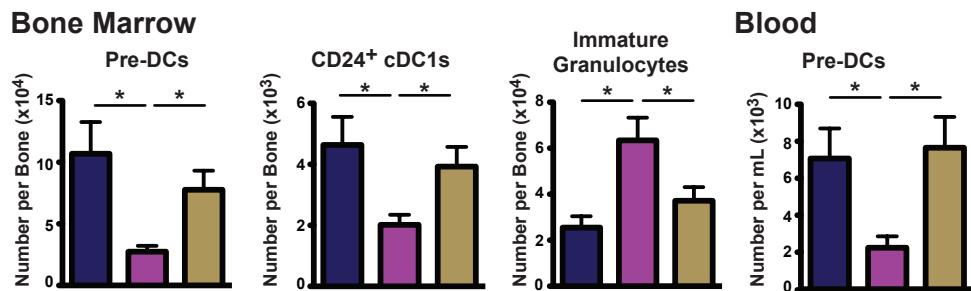
DOD Figure 5. Human breast and pancreatic cancers reduce dendritic cell progenitors and cDC1s

a. Patient bone marrow
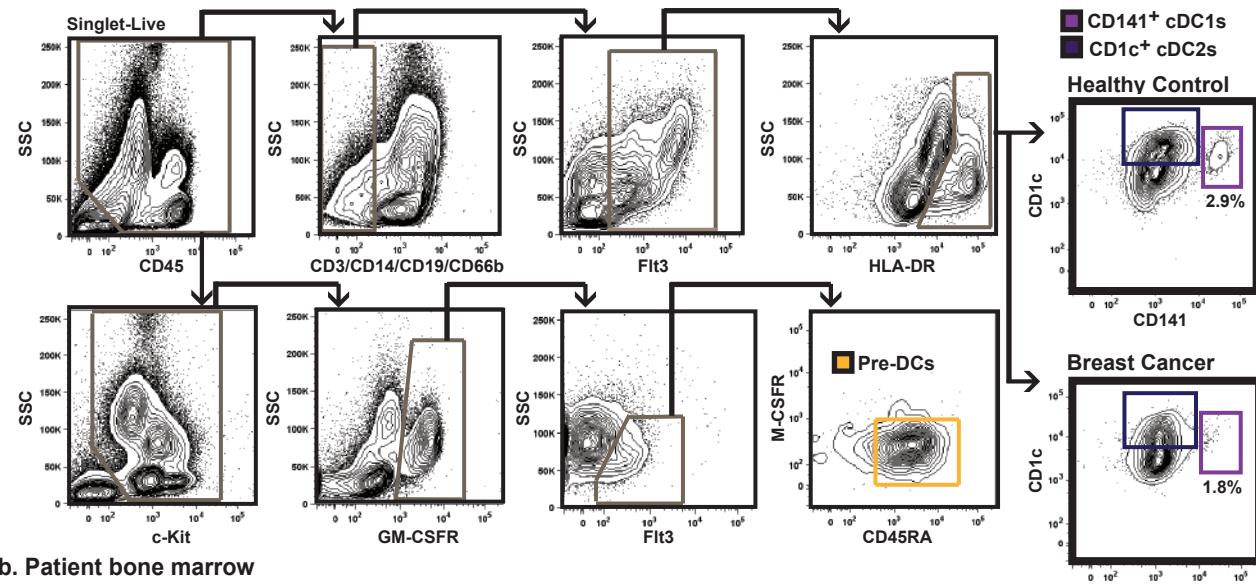
## b. Patient bone marrow
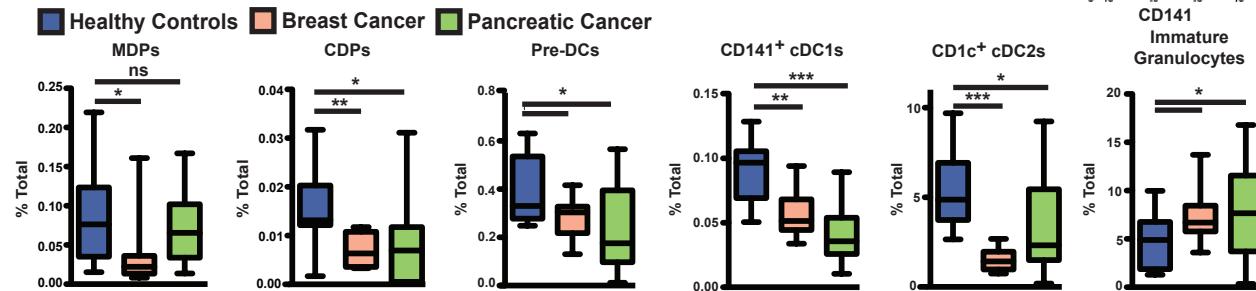

c. Patient blood
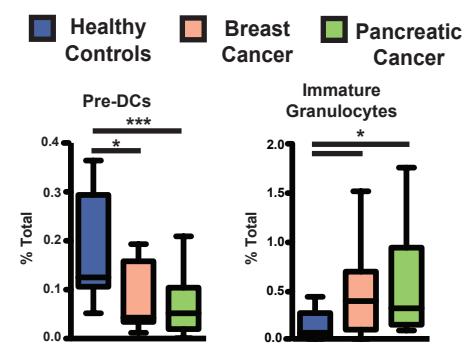
## d. Breast cancer patient pathological complete response
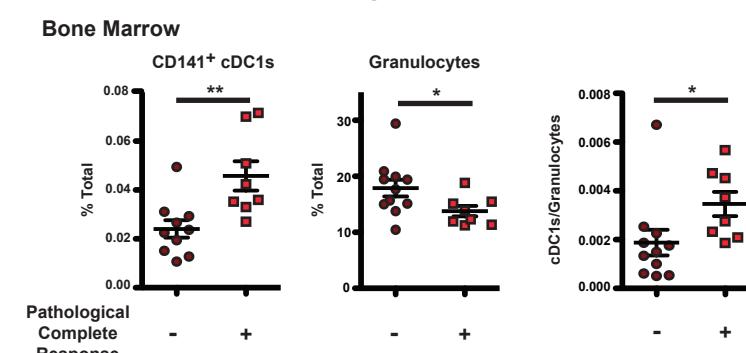
e. Breast cancer patient serum
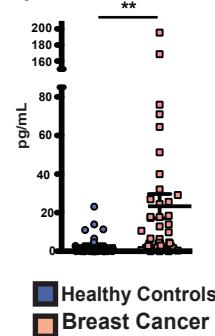
f. Patient bone marrow
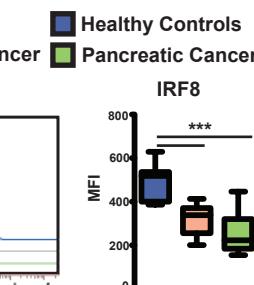
g. Breast cancer patients bone marrow
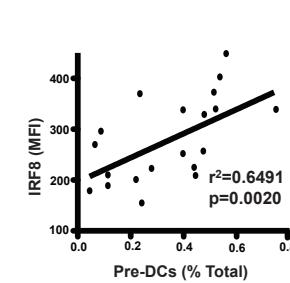
h. Cancer patient blood pre-DCs